Characterization and Mycotoxigenic Potential of *Fusarium* Species in Freshly Harvested and Stored Sugar Beet in Europe

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ABSTRACT

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Based on a 2-year field trial at two locations in Lower Saxony (Germany), 395 *Fusarium* isolates belonging to 13 species were collected from more than 3,000 sugar beet roots that were apparently healthy at harvest. In a comparative screen, subsamples were analyzed for *Fusarium* infection directly after harvest and after different storage conditions. Depending on the storage duration, a different species composition was observed. *F. redolens* was predominant in freshly harvested beets, while *F. culmorum*, *F. cerealis*, and *F. graminearum* comprised 50.0% (2006)

and 84.8% (2007) of the *Fusarium* mycoflora of sugar beets subjected to long-term pile storage. Randomly selected isolates of all species detected were tested for pathogenicity to sugar beet, but only isolates of *F. graminearum* and *F. sambucinum* caused severe root symptoms. Overall, 34 isolates of all species detected were characterized for their mycotoxin profile in rice culture to determine potentially produced toxins for future analysis of sugar beet. A total of 26 *Fusarium* mycotoxins were detected by liquid chromatography-tandem mass spectrometry, including trichothecenes, zearalenone, and especially high amounts of beauvericin, enniatins, and moniliformin. Further work is required to analyze the natural occurrence of these mycotoxins in sugar beet.

Fusarium spp. can cause yield and quality reduction in many food and feed crops, an issue best known for wheat and maize. Besides the economic losses due to *Fusarium* head blight (FHB) and *Fusarium* ear rot (69,70), mycotoxin contamination is of great concern for human and animal health (3,16). In Germany as well as in many other Central European countries, *F. graminearum* (teleomorph *Gibberella zeae*) is the predominant *Fusarium* spp. in wheat followed by *F. culmorum* and other species (7,47).

Fusarium spp. can also cause infections in sugar beet (*Beta vulgaris* L.) resulting in reduced root yield and sucrose concentration (31). A foliar yellowing and wilting caused by *F. oxysporum* f. sp. *betae* was first described in 1931 (58). Since then, *Fusarium* yellows and *Fusarium* root rot have become known in North America (32,51). Additional species have been identified that cause damage to sugar beet in the United States, including *F. acuminatum*, *F. avenaceum*, *F. culmorum*, and *F. graminearum* (28,29). In Europe, *Fusarium* is known to occasionally cause seedling damping-off or storage rot in sugar beet (50). Although neither *Fusarium* yellows nor *Fusarium* root rot have been described in commercial European sugar beet growing fields, several *Fusarium* spp. were frequently isolated from beets displaying root rots or leaf symptoms (38,53,59). Nevertheless, when tested under controlled conditions, only a few of these isolates were pathogenic (53), suggesting that they co-existed with other pathogens or were secondary invaders. Besides saprotrophic colonization, endophytic growth of *Fusarium* spp. has been recorded for various plant genera and species (40), including weed beet (35) and the well-known maize/*F. verticillioides* pathosystem (2).

Storage is an important postharvest stage of many crops during which they are exposed to different microorganisms. For example, the mycoflora of wheat is classified into field fungi (*Alternaria*, *Cladosporium*, *Bipolaris*, and *Fusarium*) and storage fungi

(*Aspergillus*, *Penicillium*), that either invade seeds before harvest or cause spoilage of stored grain (12). However, this classification cannot be strictly applied to sugar beet. Unlike wheat grains, sugar beets are not free of soil when harvested. Due to current harvesting techniques, soil losses average 9 t/ha in Europe (52). This soil adheres to the beets in outdoor storage piles and is a potential inoculum source. Moreover, mechanical damage that occurs during harvest (taproot breakage, chopping of leaves and crown) potentially provides pathways for the entry of saprotrophic organisms. Sugar beet also has a different postharvest physiology than wheat: while the moisture content of stored wheat grain is usually below 15% (12), the water content of sugar beet does not fall below 70% during storage (34). Therefore, the isolation of *Fusarium* spp. from stored beets is not surprising. In fungal-invaded stockpile-beets in Minnesota, nine different *Fusarium* spp. were detected; with 23 out of 55 isolates, *F. equiseti* was most frequently isolated (5).

However, the impact of *Fusarium* on stored sugar beet has yet to be completely investigated. A clear understanding of the presence of *Fusarium* spp. and their associated pathogenicity and mycotoxigenic potential is critical for the development of strategies for monitoring and managing diseases as well as mycotoxin contamination in sugar beet. The purposes of this study were to (i) determine *Fusarium* spp. present in sugar beet directly after harvest and during storage, (ii) characterize the pathogenic potential of randomly selected isolates of all species detected in the field trial, and (iii) determine targets for future mycotoxin analysis of sugar beet and sugar beet products by mycotoxin profiling of rice cultures. This study provides a comprehensive overview of *Fusarium* spp. and their potential mycotoxin production in freshly harvested and stored sugar beet in Europe.

MATERIALS AND METHODS

Experimental design and storage conditions. *Fusarium*-susceptible sugar beet 'Fabella' (Syngenta Seeds, Landskrona, Sweden) was sown at two locations near Göttingen, Lower

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Saxony, Germany. The locations studied are known for occasional outbreaks of FHB in wheat; however, in sugar beet no *Fusarium*-caused diseases have been observed in previous cultures. The sugar beets were grown using standard agronomic practices; fertilizer and pesticides were conventionally applied based on the recommendation of the local extension service. The field trial was integrated in a 3-year wheat-dominated crop rotation. In 2006 and 2007, a total of 1,240 and 1,800 sugar beets, respectively, were randomly harvested by hand from approximately 1,800 m² each at both locations. After harvest, subsamples (Table 1) were either used directly for fungal isolation ("freshly harvested") or stored as described below for 4, 12, or 16 weeks. According to current agricultural practice, adhering soil was not removed until processing. One subsample of the beets was stacked outside exposed to the weather in a heap, simulating pile storage for 16 weeks. No temperatures below 0°C were recorded during storage; therefore no fleece-coverage was used. To investigate the basic influence of storage without additional external influences (e.g., air dispersal of spores, rain, varying temperature, and humidity) on colonization of sugar beet, the remaining beets were stored in a controlled environment at 4°C for 4 and 12 weeks.

Isolation and species identification. The sugar beets were thoroughly washed until no adhering soil was visible. After that, two small pieces (diameter ≈0.5 cm) were removed from the center of each beet, surface-disinfected (30 s in 1.5% NaOCl, rinsed twice in sterile distilled water), blotted dry on sterile filter paper, and placed on potato dextrose agar (PDA, AppliChem, Darmstadt, Germany) supplemented with streptomycin (200 mg/liter) in plastic petri dishes. Dishes were incubated at 22°C in the dark and evaluated daily for 3 weeks for mycelial growth. Besides *Fusarium* spp., various other fungi (i.e. *Aspergillus* spp., *Penicillium* spp.) were detected but not characterized further. Beets were considered to be infected if at least one tissue piece revealed *Fusarium* growth. Pure cultures were obtained either by hyphal tip transfer or dilution plating (41), depending on the growth rate. Species identification was based on morphological traits of 3-week-old cultures grown on Spezieller Nährstoffarmer Agar (SNA) (44) at 25°C under continuous near ultra violet light (23,41). All *Fusarium* isolates were stored as spore suspensions in 15% glycerol at -80°C in the culture collection of the Institute of Sugar Beet Research (Göttingen, Germany). Morphological species identification was confirmed by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) of *tef1* partial sequences obtained with specific primers EF-1 and EF-2

(46) according to Nitschke et al. (45). DNA extraction, PCR, and restriction enzyme digestion of PCR products were conducted as described therein. Additionally, PCR products from isolates used for pathogenicity tests and mycotoxin analysis were sequenced by Eurofins (Hamburg, Germany). Sequences were checked for similarity against the NCBI and the FUSARIUM-ID (21) databases and submitted to NCBI GenBank sequence database (accession nos. HQ702566 to HQ702600).

Preparation of inoculum. One randomly chosen isolate of each *Fusarium* spp. isolated from the field trials was tested for pathogenicity to sugar beet in greenhouse experiments: *F. avenaceum* isolate O84 (culture collection no. DSM 23359), *F. cerealis* O45 (DSM 23356), *F. culmorum* O29 (DSM 23354), *F. equiseti* O19 (DSM 23353), *F. graminearum* O14 (DSM 23352), *F. oxysporum* O38 (DSM 23355), *F. proliferatum* O83 (DSM 23358), *F. redolens* O1 (DSM 23351), *F. sambucinum* O89 (DSM 23363), *F. solani* O85 (DSM 23360), *F. sporotrichioides* O87 (DSM 23362), *F. tricinctum* O63 (DSM 23357), and *F. venenatum* O86 (DSM 23361). These isolates have been deposited at the German Collection of Microorganisms and Cell Cultures (DSMZ, Braunschweig, Germany). *F. oxysporum* f. sp. *betae* isolate Fob13, previously described to cause Fusarium yellows (30) from Oregon, was used as positive control. For inoculum preparation, two plugs (diameter 0.5 cm) of fungal hyphae were transferred from the edge of an actively growing culture on PDA to 250 ml of SN broth (same ingredients as SNA [44] except for the agar). Flasks were incubated at 20°C under normal daylight conditions on a laboratory shaker (100 rpm). After 2 weeks, the spore concentration was determined with a hemacytometer and adjusted to approximately 5 × 10⁴ conidia/ml with sterile distilled water.

Plant material and inoculation. Noncoated seeds of a *Fusarium*-susceptible sugar beet hybrid (KWS Saat-AG, Einbeck, Germany) were sown in pots filled with sterilized standard potting mixture (75% sandy clay and 25% sand). Plants were grown in the greenhouse at 22/18°C (day/night) with a photoperiod of 12 h. Ten plants per treatment were inoculated and arranged in a completely randomized design. The experiment was repeated once and the two trial set-ups were analyzed together. Inoculation was performed 5 weeks after sowing (five to six leaf stage), according to a protocol of Hanson and Hill (29) with slight modifications. The beets were carefully removed from soil, washed, and inoculated by dipping roots in a spore suspension for 8 min. Spores were kept in suspension by use of a magnetic stirrer at low speed. Control plants were dipped in sterile water. Inoculated plants

TABLE 1. *Fusarium* species isolated from sugar beets subjected to different storage conditions in 2006 and 2007

Species	2006					2007					2006/07 Σ
	Storage duration (weeks) ^a					Storage duration (weeks) ^a					
	0	4	12	16	Σ	0	4	12	16	Σ	
<i>F. avenaceum</i>	—	—	—	—	—	—	—	1	—	1	1
<i>F. cerealis</i>	—	—	4	8	12	—	1	—	28	28	40
<i>F. culmorum</i>	6	2	29	24	61	4	6	7	30	47	108
<i>F. equiseti</i>	18	10	—	12	40	5	5	—	—	10	50
<i>F. graminearum</i>	—	1	8	3	12	—	—	—	9	9	21
<i>F. oxysporum</i>	5	3	—	1	9	7	2	—	—	9	18
<i>F. proliferatum</i>	—	—	—	—	—	—	1	—	—	1	1
<i>F. redolens</i>	29	17	5	6	57	29	15	4	2	50	107
<i>F. sambucinum</i>	—	—	—	—	—	—	—	—	2	2	2
<i>F. solani</i>	1	—	—	—	1	—	—	—	—	—	1
<i>F. sporotrichioides</i>	—	—	—	—	—	—	1	—	—	1	1
<i>F. tricinctum</i>	4	5	—	15	24	3	4	5	6	18	42
<i>F. venenatum</i>	—	—	—	1	1	—	—	—	2	2	3
Σ	63	38	46	70	217	48	35	17	79	178	395
Number of beets tested	500	120	120	500	1,240	500	400	400	500	1,800	3,040
Number of beets infected	61 (12.2%)	36 (30%)	45 (37.5%)	69 (13.8%)	211 (17%)	47 (9.4%)	34 (8.8%)	15 (3.8%)	78 (15.6%)	174 (9.7%)	385 (12.7%)

^a Storage for 4 and 12 weeks was conducted at 4°C under controlled conditions, storage for 16 weeks outside in a pile.

were transferred individually to 0.5-liter pots, returned to the greenhouse, and capped with plastic foil to reduce transplant shock. The foil was removed after 7 days and the temperature set to 26/22°C (day/night). Plants were evaluated weekly for the occurrence of foliar symptoms. At 9 weeks after inoculation, plants were harvested, washed, and root symptoms rated according to a 0 to 5 scale: 0 = plant healthy, 1 = single discolored spots in nearly healthy beet, 2 = few discolored spots, tissue without necrosis, 3 = discoloration and single necroses in tissue, shrunk beets, 4 = severe damaged beets with necroses but plants still alive, and 5 = tissue completely necrotic, plant lost. Reisolation and species identification followed the same protocol as described above.

Mycotoxin analysis. Thirty-four *Fusarium* strains (Tables 2 and 3) representing all species detected in the field trials were randomly chosen for mycotoxin analysis. Mycotoxin production was determined on autoclaved rice grains using a slight modification of the protocol of Greenhalgh et al. (25). Briefly, after the addition of 50 g of rice grains (Uncle Ben's long grain rice) and 70 ml of sterile distilled water to 500-ml Erlenmeyer flasks, the rice was incubated at room temperature overnight. The flasks were sealed, autoclaved twice (121°C, 15 min) and inoculated with two plugs each (0.5 cm diameter) of an actively growing culture on PDA. Flasks were incubated at 22°C in the dark. For the first 3 days after inoculation, the flasks were shaken once daily to ensure even distribution of inoculum. After 4 weeks, rice cultures were freeze-dried and ground to a fine powder with a laboratory blender. Three flasks per isolate were inoculated, grown, and ground separately. Since the purpose was to determine toxin classes produced and not their absolute quantity, replicates were not individually analyzed, but a homogeneous mixture of the three flasks was prepared. Samples were stored at -20°C until analyzed at the Center for Analytical Chemistry, Department for Agrobiotechnology (IFA, Tulln, Austria). All samples were tested

for deoxynivalenol (DON), 3- and 15-acetyldeoxynivalenol (ADON), nivalenol (NIV), fusarenon X (FUS X), diacetoxyscirpenol (DAS), monoacetoxyscirpenol (MAS), neosolaniol (NEO), HT-2 toxin, T-2 toxin, zearalenone (ZEA), α - and β -zearalenol (ZOL), fumonisin (FUM) FB₁, FB₂, and FB₃, beauvericin (BEAU), enniatin (ENNI) A, A1, B, B1, and B2, moniliformin (MON), equisetin (EQUI), 2-amino-14,16-dimethyloctadecan-3-ol (2-AOD-3-ol), and avenacin Y (AVENA Y) using a liquid chromatography-tandem mass spectrometry (LC-MS/MS)-based method which has been described previously (62,63,68).

In brief, 5 g of the mixed sample was extracted with 20 ml of a solvent mixture (acetonitrile/water/acetic acid 79:20:1, vol/vol/vol) on a rotary shaker for 90 min. The extract was diluted with the same volume of dilution solvent (20:79:1 acetonitrile/water/acetic acid, vol/vol/vol) and 5 μ l of this mixture was directly applied for LC-MS/MS. For samples containing very high ENNI and BEAU concentrations, dilutions (1:200 and 1:10,000) were reanalyzed. External calibration was performed with a liquid multitoxin standard freshly prepared prior to spiking (62,63,68). A blank sample was spiked at one concentration level in triplicate. When recovery was out of range ($100 \pm 10\%$), results were mathematically corrected. Recovery rates, standard deviation, and limits of detection (LODs) were calculated (Tables 2 and 3).

Data analysis. Statistical analysis of the pathogenicity test was performed with SAS (version 9.1; SAS Institute, Cary, NC). Single plant scores ($n = 20$) were used for the comparison of root symptoms caused by the different isolates. As the data did not fulfill the conditions of normality, the relative effect (RE) for each plant was calculated and an analysis of variance-type statistic (ATS) was performed. The REs were the basis for the following non-parametric test for factorial designs as described by Brunner and Munzel (8) and Shah and Madden (55). For multiple comparison between treatments, least square means for the ranks of marginal effects were computed (adjustment = Tukey-Kramer) and the SAS

TABLE 2. Mycotoxins^a (mg/kg) produced by tricothecene producing *Fusarium* spp. isolated from sugar beet in autoclaved rice after 4 weeks incubation at 22°C

Isolate	Type B tricothecenes					Type A tricothecenes					Zearalenon derivatives			Others	
	DON	3-ADON	15-ADON	NIV	FUS X	DAS	MAS	NEO	HT-2	T-2	ZEA	β -ZOL	α -ZOL	BEAU	EQUI
<i>F. cerealis</i>															
O41	—	—	—	—	—	—	—	—	—	—	2.1	0.1	0.01	—	—
O43	—	—	—	1.9	4.6	—	—	—	—	—	—	—	—	—	—
O45	—	—	—	0.04	0.05	—	—	—	—	—	0.06	0.01	0.01	—	—
<i>F. culmorum</i>															
O27	0.3	2.4	—	—	—	—	—	—	—	—	398	0.4	0.9	—	—
O28	—	0.03	—	—	—	—	—	—	—	—	0.3	0.05	0.02	—	—
O29	0.01	0.03	—	0.02	0.2	—	—	—	—	—	—	—	—	—	—
O64	222	1,870	290	1.1	8.9	—	—	—	—	—	1,320	5.1	2.8	—	—
O65	0.1	0.6	—	—	—	—	—	—	—	—	174	1.6	0.4	—	—
<i>F. equiseti</i>															
O19	—	—	—	0.05	—	0.2	0.2	0.2	—	—	—	—	—	—	1,050
O20	—	—	—	—	—	5.8	1.1	—	—	—	—	—	—	—	677
O90	—	—	—	0.5	0.1	0.2	0.2	—	—	—	—	—	—	0.2	765
<i>F. graminearum</i>															
O11	0.6	0.1	1.9	—	—	—	—	—	—	—	5.5	8.7	0.5	—	—
O13	0.2	0.02	0.5	—	—	—	—	—	—	—	6.3	1.0	0.1	—	—
O14	1.7	0.1	4.2	—	—	—	—	—	—	—	437	2.6	0.6	—	—
<i>F. sambucinum</i>															
O88	—	—	—	—	—	0.5	—	0.2	—	1.0	—	—	—	0.05	—
O89	—	—	—	—	—	3.2	0.6	85.8	16.9	80.8	—	—	—	2.0	—
<i>F. sporotrichioides</i>															
O87	—	—	—	—	—	0.8	0.3	25.9	14	57.5	—	—	—	0.3	—
<i>F. venenatum</i>															
O86	0.4	—	—	0.9	0.4	34	11.2	1.0	—	1.6	—	—	—	0.1	—
REC (%) ^b	97.7	101	96.7	80.3	100	114.6	105.3	120.3	108.1	106.3	105.4	104	104	108.9	85.8
SD (%)	3.3	1.4	12.6	1.7	13.1	9.1	22.1	4.7	11.4	15	1.6	9	2.3	6.3	3.9
LOD (mg/kg) ^c	0.008	0.008	0.12	0.012	0.016	0.0024	0.016	0.012	0.02	0.008	0.016	0.0024	0.008	0.0004	0.12

^a DON, deoxynivalenol; NIV, nivalenol; 3-ADON, 3-acetyldeoxynivalenol; 15-ADON, 15-acetyldeoxynivalenol; DAS, diacetoxyscirpenol; MAS, monoacetoxyscirpenol; NEO, neosolaniol; HT-2, HT-2 toxin; T-2, T-2 toxin; ZEA, zearalenone; ZOL, zearalenol; BEAU, beauvericin; and EQUI, equisetin.

^b REC, recovery rate, when out of the range of 100 ± 10 , results were corrected.

^c LOD, limit of detection. — indicates that mycotoxin amounts are below the LOD.

macro MULT (<https://www.unihohenheim.de/bioinformatik/beratung/tools/macros/sas/macros/mult.sas>) was used to assign letters. The estimated relative treatment effects with appropriate confidence limits for the REs of the single plants were calculated with the SAS macros LD_CI (http://www.ams.med.unigoettingen.de/de/sof/ld/LD_CI.SAS) and OWL (<http://www.ams.med.unigoettingen.de/amsneu/download/longit/OWL.SAS>), respectively.

RESULTS

Species composition in freshly harvested and stored sugar beet. No symptoms comparable to Fusarium yellows or Fusarium root rot were observed at the two locations studied, either during the growing season or at the time of harvest. Although the beets were apparently healthy at the beginning of the storage trials, some of them had developed a severe storage rot after 12 or 16 weeks of storage in both controlled environment and outside pile assays. Discoloration and rotting tissue were concentrated predominantly at the taproot and in the area of the chopped crown.

Overall, 395 *Fusarium* isolates were recovered and taxonomically assigned to 13 species, seven of them occurring in higher frequencies (Table 1). On average, 12.7% (range of 3.8 to 37.5%) of the beets were considered to be infected. The majority of the beets revealed only a single *Fusarium* spp., but a few were infected with at least a second one. No continuous increase of the infection rate with increasing storage duration was noted, but a different species composition was observed depending on the storage duration (Table 1). In both years, *F. redolens* was the species most frequently isolated from freshly harvested beets (46 and 63% of all isolates in 2006 and 2007, respectively) followed by *F. equiseti*, *F. oxysporum*, *F. culmorum*, and *F. tricinctum*. In contrast, *F. culmorum* was the predominant species in beets subjected to long-term storage in piles. When summarizing cereal-pathogenic species *F. culmorum*, *F. cerealis* (syn. *F. crookwellense*), and *F. graminearum*, they collectively constituted 50% (2006) and 84.8% (2007) of all isolates recovered. *F. grami-*

nearum and *F. cerealis* were not detected in beets directly after harvest. In contrast, *F. oxysporum* was, with one exception, only isolated from beets freshly harvested or subjected to short-term storage.

Similar results were obtained from sugar beets stored under controlled conditions (Table 1). *F. redolens* was still the most prevalent species in beets subjected to short-term storage at 4°C for 4 weeks (45 and 43%, respectively), while after 12 weeks, *F. culmorum* was again the predominant species (43 and 41.2%, respectively).

Pathogenicity test. Foliar symptoms were restricted to the beets inoculated with the positive control *F. oxysporum* f. sp. *betae* (isolate Fob13), with the single exception of one plant inoculated with *F. sambucinum* (O89) that showed complete wilting while exhibiting a severe root rot. In plants exhibiting foliar symptoms, the first leaf symptoms were observed after a latency of 4 weeks. Initially, one-sided interveinal chlorosis followed by yellowing of the whole lamina was most prominent. Later, necrosis, downward twisting of leaves, and wilting of the plant was observed.

Root symptoms were more evenly distributed across the trial (Fig. 1). Statistically, the different isolates tested could be divided into three groups: *F. oxysporum* f. sp. *betae*, German isolates significantly differing from the water control, and German isolates not differing from the water control. Fob13 caused the most severe root symptoms with an average disease score (ADS) of 3.65. The relative effect of this isolate differed significantly from all German isolates tested (Fig. 1). Nevertheless, *F. sambucinum* (O89, ADS 1.7) and *F. graminearum* (O14, ADS 1.15) were also able to cause symptoms which were rated in categories 4 and 5, respectively. Cross sections displayed intense discoloration, necroses of vascular elements, and stunting of the beets. However, most sugar beets inoculated with isolates belonging to other species exhibited, if any, a single or a few brownish spots within the central cylinder. Only one plant inoculated with O38 (*F. oxysporum*) showed a brownish discoloration of the whole central

TABLE 3. Mycotoxins^a (mg/kg) produced by trichothecene nonproducing *Fusarium* spp. isolated from sugar beet in autoclaved rice after 4 weeks incubation at 22°C

Isolate	Fumonisin			Cyclohexadepsipeptides						Others		
	FB ₁	FB ₂	FB ₃	BEAU	ENNI A	ENNI A1	ENNI B	ENNI B1	ENNI B2	MON	2-AOD-3-ol	AVENA Y
<i>F. avenaceum</i>												
O84	–	–	–	–	21.1	385	3,000	2,400	78.8	700	3,090	4,790
<i>F. oxysporum</i>												
O38	–	–	–	73.2	1,300	624	6.9	118	0.5	0.5	–	–
O40	–	–	–	40	37.4	29.1	0.8	9.4	0.02	3.8	–	–
<i>F. proliferatum</i>												
O83	6.1	1.4	5.2	0.3	–	–	–	–	–	2.2	–	–
<i>F. redolens</i>												
O1	–	–	–	163	13.7	12.1	0.5	3.5	0.01	–	–	–
O4	–	–	–	17.3	2.2	1.0	0.01	0.1	–	–	–	–
O8	–	–	–	50.2	0.8	1.3	0.1	0.3	0.01	0.2	–	–
O37	–	–	–	83.2	0.6	0.3	0.2	0.6	0.1	0.8	–	–
O52	–	–	–	1,120	–	–	–	–	–	47.9	–	–
O54	–	–	–	1,580	17.4	15.8	1.1	6.3	0.03	76.4	–	–
O55	–	–	–	624	–	–	–	–	–	36.1	–	–
O57	–	–	–	948	–	–	–	–	–	17.7	–	–
<i>F. solani</i>												
O85	–	–	–	0.01	0.01	0.01	–	–	–	–	–	–
<i>F. tricinctum</i>												
O32	–	–	–	–	190	478	119	400	3.4	1.3	336	–
O35	–	–	–	–	199	880	356	1,090	8.3	1.4	1,160	2,250
O63	–	–	–	–	310	1,350	967	2,340	37.4	4.9	847	397
REC (%) ^b	82.2	100.0	84.5	108.9	96.1	90.7	127.1	112.5	105.5	88.7	85.2	n.d.
SD (%)	9.1	5.7	10.1	6.3	9.4	6.1	0.9	8.1	9	6.3	11.2	n.d.
LOD (mg/kg) ^c	0.064	0.016	0.032	0.0004	0.00024	0.0004	0.00004	0.0005	0.00024	0.016	0.024	0.04

^a BEAU, beauvericin; ENNI, enniatin; MON, moniliformin; 2-AOD-3-ol, 2-amino-14,16-dimethyloctadecan-3-ol; and AVENA Y, avenacin Y.

^b REC, recovery rate, when out of the range of 100 ± 10, results were corrected.

^c LOD, limit of detection. – indicates that mycotoxin amounts are below the LOD.

cylinder. Isolates of the two most frequently isolated species, *F. redolens* (O1, ADS 0.7) and *F. culmorum* (O29, ADS 1.05), differed significantly from the water control. Nevertheless, more than 50% of the plants inoculated with these two species were rated as 0 or 1. With one exception, root symptoms were limited to the internal tissues. The only plants with symptoms on the root surface were those inoculated with *F. avenaceum* (O84); some of the beets had black necrotic lesions on the crown, restricted to the cortex. Although several inoculated beets did not show any root symptoms, reisolation of the inoculated species was possible (data not shown).

Mycotoxigenic potential of *Fusarium* spp. isolated from sugar beet. Mycotoxin production of *Fusarium* spp. on autoclaved rice grains is shown in Tables 2 and 3 for trichothecene-producing and nonproducing *Fusarium* isolates, respectively. Generally, the results confirmed the species designation. However, a few unexpected mycotoxin combinations were found. With the exception of O64, all cultures of *F. cerealis*, *F. culmorum*, and *F. graminearum* revealed rather low amounts of type B-trichothecenes, while high amounts of ZEA and its derivatives were detected (Table 2). Simultaneous production of DON- and NIV-derivatives was observed for isolates of *F. culmorum* (O29, O64) and *F. venenatum* (O86). The latter also showed the broadest range of mycotoxins in this study, including type A- and B-trichothecenes (DON, NIV, FUS X, DAS, MAS, NEO, and T2) as well as BEAU. Type A-trichothecenes were also detected in cultures of *F. equiseti*, *F. sambucinum*, and *F. sporotrichioides* (Table 2). The only isolate which produced FUM in detectable amounts was taxonomically assigned to *F. proliferatum* (Table 3). Mycotoxin production by *F. redolens* and *F. oxysporum* was restricted to the cyclohexadepsipeptides BEAU and ENNI as well as MON. Although toxin production varied between the different isolates of *F. redolens*, BEAU was much more prevalent than ENNI. On the contrary, in cultures of *F. oxysporum* higher amounts of ENNI were detected (Table 3). Cultures of *F. avenaceum* and *F. tricinatum* produced not only high levels of ENNI and MON, but also significant amounts of 2-AOD-3-ol and AVENA Y. In addition to the results shown in Tables 2 and 3, trace amounts of ENNI just above the LOD were found in some cultures of *F.*

cerealis, *F. culmorum*, *F. equiseti*, *F. sambucinum*, *F. sporotrichioides*, and *F. venenatum*, and traces of 3-ADON were determined in a culture of *F. redolens* (data not shown).

DISCUSSION

Until now, at least 17 *Fusarium* spp. have been isolated from sugar beet (5,29,45,51,53). With the detection of 13 different species in sugar beets from two locations, the results of this study support these prior reports. Furthermore, the findings of the present study also indicate that shifts in the frequency of species may occur during storage.

The relatively low isolation frequency of *F. oxysporum*, the main causal agent of the severe yield and quality losses in the United States (29,31,32), was quite surprising. Although this species is a ubiquitous soil inhabitant, it does not seem to be relevant in Germany. The percentage of individual species in the different storage treatments varied between 2006 and 2007. However, the two species *F. redolens* and *F. culmorum* were dominating depending on the storage duration. *F. redolens*, which is a practically unknown in agricultural crops other than potato (49) and horticultural crops (4,22,24), proved to be the most frequently isolated species in freshly harvested beets. In contrast, the other frequently isolated species *F. cerealis*, *F. culmorum*, *F. equiseti*, *F. graminearum*, and *F. tricinatum* are known to be part of the FHB complex in Europe (7,47) and, therefore, widespread in wheat-growing areas. The locations studied here have a history of wheat/sugar beet-based crop rotations for decades. It may be assumed that a *Fusarium* population typical for these crops has been established because of the longevity of fungal propagules (42,56). Other *Fusarium* spp. might prevail in sugar beets grown in different rotations. However, while crop rotation is one of the most important factors for FHB (17), until now, the influence of crop rotation on infection rate and *Fusarium* species composition in sugar beet is unknown.

F. culmorum and *F. graminearum* are best known for their pathogenicity on wheat. However, *F. graminearum* was also identified as a minor cause of Fusarium yellows in sugar beet in some regions of the United States (28), while Hull (33) described

Fig. 1. Results of greenhouse pathogenicity test of *Fusarium* spp. in sugar beet ($n = 20$). Beet symptoms are rated according to a 0 (healthy) to 5 (beet completely necrotic) scale. Isolates with the same letter are not significantly different ($\alpha = 0.05$).

F. culmorum as a secondary pathogen in Europe, attacking sugar beets when their viability is lowered due to drought or heart rot. Subsequently, the alternating frequencies of *F. redolens* on the one hand and *F. culmorum*, *F. cerealis*, and *F. graminearum* on the other hand, in freshly harvested in comparison with pile-stored beets, can likely be explained by saprotrophic colonization via injuries caused during harvest. This hypothesis is supported by the observation of root rot symptoms around the chopped crown and the taproot of previously healthy beets after 16 weeks of storage. The same species shift from *F. redolens* to *F. culmorum* was detected in the controlled storage trials. Due to the observation of saprotrophic colonization under controlled conditions, it can be assumed that the adhering soil is the main important factor for postharvest *Fusarium* infection in sugar beet. External factors (e.g., air dispersal of spores, rain, varying humidity, and temperature) do not appear to be crucial for infection after the beets are harvested.

Although no foliar or root symptoms were observed either during the growing season or at harvest, 12.2 (2006) and 9.4% (2007) of freshly harvested and healthy-looking beets, respectively, were infected with *Fusarium*. The infection rate of the beets examined was probably much higher, as the isolation method was based on just two tissue pieces from the center of the beet. Unequal distribution of fungal structures might have led to some isolates being missed during isolation. According to Petrini (48), all organisms colonizing internal plant tissues, without causing apparent harm to the host, are endophytes. Nevertheless, this definition only refers to the time of isolation. Changes in plant physiology and biotic or abiotic stress factors may turn a former endophyte into a pathogen (54). Therefore, the purpose of the pathogenicity test was to determine the pathogenic potential of randomly selected *Fusarium* strains which were isolated from initially healthy-looking sugar beets. Under the given greenhouse conditions, most inoculated beets did not reveal any symptoms while a few exhibited single brownish spots within the root tissue. Reisolation was possible from completely healthy-looking beets (data not shown). This strongly indicates the low pathogenic potential of the isolates tested. However, two isolates of *F. sambucinum* (O89) and *F. graminearum* (O14) were able to cause root symptoms which were comparable to the positive control *F. oxysporum* f. sp. *betae*. But due to the nonlinear rating scale and the weak nonparametric test, their disease scores did not differ significantly from other far less aggressive isolates like O1 (*F. redolens*) and O84 (*F. avenaceum*). While *F. graminearum* has previously been described to cause *Fusarium* yellows in sugar beet (28), *F. sambucinum* has been isolated from sugar beet (51), but is more familiar as a potato dry rot pathogen (18).

Prior studies have shown that biotic and abiotic factors can affect symptom development in sugar beet (30,32,33). This is supported by past studies that found 21% (29) and 25% (51) of the *Fusarium* strains isolated from *Fusarium* yellows affected sugar beet roots under controlled conditions. Therefore, it has to be considered that pathogenic isolates might be missed by testing only one isolate of each species. However, the abovementioned results as well as the absence of symptoms in the field and at harvest in combination with isolation frequencies of about 10% suggest constant background colonization with endophytic *Fusaria*. Therefore, not all *Fusarium* frequently isolated from sugar beets displaying root rot symptoms in the field (38,53,59) have to be the causal agent of the disorder.

As the didactic distinction between endophytes, pathogens, or saprobes does not influence the isolates ability to produce mycotoxins (2,40), this topic has an impact for both *Fusarium* epidemic and nonepidemic areas of sugar beet production. Only a few studies are available referring to mycotoxin contamination in sugar beet and sugar beet products. Production of ZEA was reported by Bosch et al. (6) to be 10 times higher in rice cultures (0.7 to 1.9 mg/kg) than in incubated sugar beet slices, which

indicated a lower production in planta. On the other hand, ZEA was found in concentrations up to 4.65 mg/kg in sugar beet fibers and sugar beet-based feeds (5,57).

Before implementing an expensive and time-consuming screening for mycotoxins in sugar beet and sugar beet products, a survey of toxins potentially produced had to be conducted. Rice is commonly accepted as a standard medium for mycotoxin production (25,66), but it is still a natural substrate and mycotoxin background contamination cannot be excluded. In particular, it is difficult to distinguish between fungal production and background contamination for amounts just above the LOD. This most probably explains traces of 3-ADON detected in a culture of *F. redolens* as well as the traces of ENNI in several cultures including *F. cerealis*, *F. culmorum*, and *F. solani*. In vitro mycotoxin analyses can only serve qualitative purposes as there is no correlation between the amounts produced in vitro and in planta (15,19). Therefore, no predictions regarding the amounts of the different mycotoxins in sugar beet can be made. Further analyses of inoculated sugar beets have to prove if and in which concentrations the individual toxins are produced in planta.

Except for the unusual broad mycotoxin profile of *F. venenatum*, the mycotoxin profiles of sugar beet isolates are in line with the profiles described for *Fusarium* spp. from other hosts (13 and references therein). DON as well as NIV and FUS X were detected in cultures of *F. culmorum* (O29, O64) and *F. venenatum*. Concomitant production of NIV and DON has been reported for several strains of *F. graminearum* (14,39,61) and *F. culmorum* (43). *F. venenatum* as well as *F. equiseti* also revealed production of both type A- and B-trichothecenes. Current understanding of the trichothecene biosynthetic pathway in *Fusarium* indicates that coproduction of scirpenol, NIV, and DON derivatives is possible (13). As mentioned above, the *F. venenatum* isolate exhibited a very broad mycotoxin profile, which included the detection of low amounts of BEAU. This result was confirmed by an independent repetition of a separately grown culture (data not shown). The closely related species *F. tricinctum* and *F. avenaceum* (64,65) shared a similar mycotoxin profile of ENNI, MON, AVENA Y, and 2-AOD-3-ol production. The latter toxin has only recently been discovered from *F. avenaceum* cultures (67). Although structurally belonging to the sphingosine analogues like the fumonisins, the first in vitro tests revealed cytotoxic but nonceramide synthase inhibiting properties of 2-AOD-3-ol (66).

F. redolens (sometimes referred to as *F. oxysporum* var. *redolens* in older publications), was applied for the first time in a comprehensive mycotoxin profiling. Some strains of *F. redolens* have been reported to produce FB₁, FB₂, and FB₃ (1), but none of the isolates tested in this study produced amounts of these toxins above the LOD. Based on the observation that *F. redolens* was the predominate species in freshly harvested, apparently healthy beets, the low pathogenic potential and the ability of this species to produce BEAU, MON, and ENNI in high concentrations in vitro, a possible mutualistic effect can be hypothesized. BEAU and ENNI are known to have insecticidal, anthelmintic, and antibiotic functions (20,26,27,36,37,60). The natural occurrence of these *Fusarium* mycotoxins in sugar beet has yet to be determined, but if these toxins are produced in efficacious amounts in planta, there might be an advantage for the host to have endophytic colonization by this species.

Regarding the *Fusarium* species composition detected in sugar beet in this study, large amounts of trichothecenes and ZEA are not to be expected in freshly harvested and apparently healthy beets. Species capable of producing DON and ZEA only gained importance with increasing storage duration. Therefore saprotrophic colonization during storage might explain the high amounts of ZEA observed as has been shown in previous studies (5,57).

Due to the multitude of mycotoxins produced in vitro, and the high incidence of species producing BEAU, ENNI, and MON, an extensive screening is required in order to examine the impact of

mycotoxins in planta. Target toxins for future mycotoxin analysis of sugar beets and sugar beet products should at least include type B-trichothecenes, ZEA, BEAU, ENNI, and MON. Further studies are necessary to investigate the role of endophytic and saprotrophic *Fusarium* infection on sugar beet culture and mycotoxin contamination as well as the influence of agricultural factors. The latter is especially important as Burlakoti et al. (9,10,11) demonstrated that *F. graminearum* isolates collected from sugar beet were able to cause typical FHB symptoms and characteristic mycotoxin contamination in wheat. In regard of the economic impact of FHB, further studies should not only determine the influence of crop rotation on *Fusarium* infection in sugar beet but also the influence of sugar beet residues on crop rotation.

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